The Pharma Innovation Journal 2023; 12(3): 4056-4061

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(3): 4056-4061

www.thepharmajournal.com
Received: 08-01-2023
Accepted: 13-02-2023

Dr. M Sakthi Priya
Assistant Professor,
Animal Feed Analytical and Quality Assurance Laboratory,
Veterinary College and Research Institute, Namakkal,
Tamil Nadu, India

A Jagadeeswaran
Professor and Head,
Department of Veterinary Pharmacology and Toxicology,
Veterinary College and Research Institute, Namakkal,
Tamil Nadu, India

MJ Raja
Assistant Professor,
Department of Veterinary Pharmacology and Toxicology,
Veterinary College and Research Institute, Orathanadu,
Tamil Nadu, India

A Natarajan
Professor and Head,
Animal Feed Analytical and Quality Assurance Laboratory,
Veterinary College and Research Institute, Namakkal, Tamil Nadu, India

P Srinivasan
Professor and Head,
Department of Veterinary Pharmacology and Toxicology,
Veterinary College and Research Institute, Thalaivasal, Salem,
Tamil Nadu, India

Corresponding Author:
Dr. M Sakthi Priya
Assistant Professor,
Animal Feed Analytical and Quality Assurance Laboratory,
Veterinary College and Research Institute, Namakkal,
Tamil Nadu, India

A suitable substrate for the production of ochratoxin a (OTA) by *Aspergillus ochraceus*

Dr. M Sakthi Priya, A Jagadeeswaran, MJ Raja, A Natarajan and P Srinivasan

Abstract
Ochratoxin A (OTA), a known nephrotoxin is produced by *Aspergillus* and *Penicillium spp* contaminating crops. As there are numerous factors known to influence the OTA production, the present study was undertaken to explore a suitable substrate for the production of OTA under laboratory. Spores from the working culture of *Aspergillus ochraceus* MTCC No.1810 were inoculated into selected substrates like polished rice, maize, pearl millet and de-oiled cakes like groundnut oil cake and deoiled sunflower meal and toxin was estimated after seven days. The results revealed noticeable levels of OTA in deoiled sunflower meal and pearl millet. Thus deoiled sunflower meal was selected for the second phase, owing to its usage in poultry feeds at a level of 10 - 15 percent as a source of protein and subjected to toxin production for a period of 28 days. The toxin was detected at weekly intervals. The results revealed 8, 17, 25 to 58 ppb on 7, 14, 21 and 28 days respectively with the highest level detected on 28th day at a temperature of 28 °C – 32 °C. Thus deoiled sunflower meal would be a better substrate than other cereals and oil cake to stimulate OTA production among the substrates tested.

Keywords: *Aspergillus ochraceus*, mycotoxins, ochratoxin A (OTA), deoiled sunflower meal, cereals

1. Introduction
Mycotoxins are produced by various filamentous fungal species including three predominant genera *Aspergillus*, *Penicillium* and *Fusarium* that can contaminate the crops in the field. These fungal species producing toxin grow well at an optimum temperature between 10 °C and 40 °C, and at a pH range of 4-8, and at water activity levels above 0.70. However, their growth was also visible on a very dry surface (Lacey, 1991) [15]. When there was a favourable environmental condition, they were found to thrive well on any solid or liquid support. But the growth condition was found to vary for each fungal species when compared with the field and the post-harvest stages (Bhat *et al*., 2010) [5]. The quantum and the toxigenic nature of mycotoxins produced from the fungi are highly influenced by the type of substrate, moisture content, available nutrition, temperature, humidity in the surrounding environment, maturity of the fungal colony, co-occurrence of other fungi, competition from other microorganisms, stress factors, physical damage of the substrate due to insect infestation and other associated factors (Coulumbe and Coulumbe, 1993, Hendry and Cole, 1993, Viquez *et al*., 1994, Rao, 2001) [7, 11, 31, 21]. The toxins that are commonly produced by these fungi include aflatoxin, ochratoxin, fumonisin, trichothecenes and zearalenone.
Ochratoxin A produced by *Aspergillus* and *Penicillium* spp appear during storage of fresh produce and occasionally in the field crops. The toxigenic nature of OTA has been classified by the IARC as a Class 2B carcinogen (Leszkowicz and Manderville, 2007) [17] as it causes nephrotoxicity, teratogenicity and immunotoxicity, and associated tumor manifestations in human. The primary organs of toxicity of OTA are kidney followed by liver and it was found to produces acute toxicity (Lindblad *et al*., 2004) [18]. The severity of the toxic symptoms is primarily noticed at a higher level in mammals and poultry and the symptoms include loss of appetite, weight loss, decrease in production leading to disease condition such as toxic hepatitis, haemorrhage, oedema, immunosuppression, carcinoma of the liver, esophageal cancer and kidney failure (Donmez–Altunta *et al*., 2003) [8]. Even with effective culture of *Aspergillus ochraceus*, the production of toxin in sufficient quantities for the experimental studies under laboratory conditions has been challenging. Choices of substrates for growing the fungi and producing OTA needs to be explored for harvesting adequate amount of OTA for conducting toxicological studies in poultry. Suitable substrates with necessary nutrients for the growth of fungus are very essential for the production and accumulation of OTA by *A.*

ochraceus (Angel *et al*., 2004) [1].
Hence, it was very essential to identify a suitable substrate for the production of OTA under laboratory conditions and demands much toxicological and mycological research over other mycotoxins for finding out solutions to the problem (Jand *et al*., 2005) [13] that has really threatened by creating greater economic impact with reduction in productivity in livestock and poultry. Also, when more information was known about the ideal conditions and substrates promoting the growth of the fungus and toxin production, suitable preventive measures can be adopted. Hence with this objective in mind the present study was undertaken to find out the suitable substrate for the production of OTA and also to determine the effect of incubation time on the production of the toxin under laboratory.

2. Materials and Methods

2.1 Culture and Inoculum

OTA was produced using the pure culture of *Aspergillus ochraceus* MTCC No.1810. The reference culture was sub cultured in potato dextrose agar slant for 24 hours at 28 °C to obtain the working culture. The slants with potato dextrose agar were then inoculated with spores from the working culture and it was allowed for incubation for 7 days at 28 °C for spore development and subsequent substrate inoculation when required. By seven days, the culture developed a heavy crop of white conidia. The spores were then scraped loose with a loop and the slant was shaken with the spores aseptically to produce a uniform suspension of spores, and the spore suspension (0.5mL) was then used to inoculate each 50g of substrate.

2.2 Substrate Processing

Three cereals polished rice, maize and pearl millet and two oil extracted cakes groundnut oil cake and deoiled sunflower meal were selected as the substrate for the production of OTA as per the method of production of aflatoxin B1 with slight modifications (Shotwell *et al.,* 1966) [22].

2.3 Culture

Culture was done in 300 mL Erlenmeyer flasks each containing 50g of polished rice, maize, pearl millet, groundnut oil cake and deoiled sunflower meal separately. 25 mL of tap water was added to the substrate in the Erlenmeyer flasks and the mixture was kept for incubation for two hours at the room temperature of 28 ºC to 32 ºC with frequent shaking. The flasks were then autoclaved at 15 *psi* for 15 minutes and allowed to cool. Sterile water was added at 24 and 48 hours, just enough not to cause any substrate particle to adhere to one another. The entire experiment was carried out during the month of February and March with an average room temperature of 28 °C – 32 °C. The flasks were shaken vigorously every two hours to loosen the material. It was extremely important to maintain the substrate free of any compact mass as the mold growth may bind the kernels together.
At 48 hours after inoculation, the substrate showed small white areas which indicated the growth of the mold. Shortly afterwards, the substrate turned to a pale white colour (Figure 1). After the end of the 7th day, the toxin was estimated.
The substrate in which the maximum toxin was detected at the end of seven days of incubation was selected and the selected substrate was subsequently used for the production of toxin for a period of 28 days and the quantity of toxin was determined intermittently at weekly intervals.

2.4 Toxin estimation

OTA estimation was done as per the method of AOAC (1995) [2] by following the procedure of estimation of multi-mycotoxins using Thin Layer Chromatography at Animal Feed Analytical and Quality Assurance Laboratory, Veterinary College and Research Institute, Namakkal.
The toxin was extracted with acetonitrile, potassium chloride and hydrochloric acid, filtered and defatted with hexane, twice. The fat free extract was further extracted with chloroform, dried, rediluted in high purity chloroform and spotted on a precoated aluminium sheet of 0.25mm thickness (Merck, Germany) along with standards and allowed to air dry.

2.5 Preparation of OTA standard

OTA standard (procured from M/s. Sigma Aldrich) was prepared carefully in a suitable standard flask with benzene: acetic acid (99+1) mixture to give a concentration of 10µg/mL. From this stock solution, OTA solution was prepared in benzene: acetic acid to the concentration of 4µg/mL and was used as the working standard.

2.5.1 Spotting and Developing

After spotting the test sample and standards of different concentration, it was allowed for developing in an unsaturated first developing tank containing chloroform: acetone: in the ratio 88:12. For a total volume of 10 mL. When three fourth of the plate was found to be developed, the plate was carefully removed from the tank, dried well and placed perpendicular to the first step in the second developing tank containing toluene: ethylacetate: formic acid in the ratio of 50:40:10 for a total volume of 10 mL. When three fourth of the plate was found to be developed, the plate was carefully removed from the tank, dried well and viewed in a UV cabinet viewer using long wavelength (364 nm).

2.5.2 Calculation

$$\text{Ochratoxin content in µg/kg} = \frac{\text{SxCxD}}{\text{TxE}} \text{ X } 1000$$

S = Standard which was compared with the sample based on fluorescent intensity.
C = Concentration of the standard.
D = Dilution factor.
T = Sample which was compared with the standard based on fluorescent intensity.
E = Effective weight.

3. Results and Discussion

All the substrates used for OTA production *viz*., polished rice, maize, pearl millet, groundnut oil cake and de-oiled sunflower cake showed good growth of fungi observed with spore production. However, OTA was produced only in de-oiled sunflower cake and pearl millet at the level of 8 µg / kg (Figure 2) during the first seven days of incubation and no levels of toxin was detected in other substrates used *viz*., polished rice, maize and groundnut oil cake.
Thus, based on the result, de-oiled sunflower cake was chosen for further

toxin production work for a period of 28 days (Figure 3). The second growth experiment conducted with de-oiled sunflower cake for 28 days revealed 8, 17, 25 and 58 µg / kg of OTA during 7, 14, 21 and 28 days respectively with the highest level on 28th day.

As OTA was first known to be isolated from *Aspergillus ochraceus*, this species has a unique identity and since then it was being used as a model system to study OTA production (Van der Merwe *et al*., 1965a, 1965b; Lai *et al*., 1970; Tsubouchi *et al.*, 1995 and Varga *et al.*, 2000) [26-27, 16, 25, 30]. There are numerous factors which may contribute to the production of OTA, like high level of moisture in the crop either at the field condition or during post-harvest storage, extremes of temperature, high level of humidity in the environment, stress arousing due to drought conditions and infestation by the insects and other pests (Coulombe and Coulombe, 1993) [7] and a suitable substrate (Angel *et al*., 2004) [1] which favor fungal growth and enhance toxin production leading to contamination with mycotoxins. An increased quantity of OTA has previously been reported on grains such as corn or wheat (Van der Merwe *et al*., 1965a; Ciegler, 1972 and Hesseltine *et al.*, 1972) [26, 6, 12]. Although rice was naturally contaminated with *Aspergillus ochraceus*, it was not found as a suitable substrate for OTA production (Begun and Samajpati, 2000 and Trung *et al.*, 2001) [3, 24]. Hence, the absence of OTA in the cereals like rice, maize and in the de-oiled groundnut cake noticed in our survey may be attributed to the above findings *viz.*, the lack of suitable substrate, insufficient time of incubation and lack of other factors required under the natural field conditions like high moisture, a high level of drought stress or combination of more than one of these factors as well.

Thus, based on the result, with noticeable levels of OTA detected during the first seven days of incubation in de-oiled sunflower cake and pearl millet the substrate was selected. Further, de-oiled sunflower cake was chosen for toxin production for a period of 28 days by comparing the advantages and the importance as the de-oiled sunflower cake holds as an important source of raw material in feed manufacturing over the other. Thus, de-oiled sunflower cake would be an apt choice for further study as per the above discussions and the second growth experiment was conducted with de-oiled sunflower cake for 28 days. The temperature of 28 °C was optimum for the production of OTA (Trenk *et al*., 1971) [23] and the OTA formation at 37 °C was considerably lower than at 28 °C and attributed the finding to the rapid degradation of the toxin at 37 °C or decreased toxin formation at this temperature. Also, the amount of available nutrients in any substrate, water activity and oxygen are the major factors governing the growth of fungi and production of toxin. However, the possible reason for the predomination of a fungal species in one particular substrate is not known, and it is often correlated with either the species characteristics or the properties of the product. To predict the mycotoxin production in any substrate, knowledge on the specific mycobiota of the product and data from the laboratory experiments are essential (Filtenborg *et al*., 2000) [9] The toxin profiles and the amount produced depends entirely on the ecological and processing parameters of the material (Filtenborg *et al*., 1996) [10].

Many studies reported variation in the production of OTA even among the strains of *A. ochraceus* (Hesseltine *et al*., 1972; Tsubouchi *et al*., 1995; Varga *et al*., 1996 and Van der Merwe *et al*., 1965b) [12, 25, 29, 27]. Similarly, there was a considerable variation in the production of OTA among different *Aspergillus spp.* isolates (Ciegler, 1972; Hesseltine *et al*., 1972; Varga *et al*., 2002) [6, 30]. *Penicillium nordicum* was able to produce OTA, whereas *Penicillium verrucosum* synthesized both OTA and citrinin. In bread analogue, high amount of ochratoxin A was produced by *P. verrucosum*, resulting in major variations in the toxin profiles produced by these two strains (Kokonen *et al*., 2005) [14]

The major factors why a fungal species predominate in a particular substrate with high level of toxin production and the functions associated with it are still not clear (Betina 1989: Filtenborg *et al*., 2000) [4, 9] Among the different factors associated, the substrate was found to contribute to a larger extent in terms of production and as well as accumulation of OTA and the bee pollen was found as a suitable and good substrate for the characterization of different strains of OTA producing *A. ochraceus* for the maximum period of 28 days. Bee pollen was a rich source of the free amino acids and speculated a positive correlation between amino acid composition and OTA production and the impact of time duration on toxin production (Angel *et al*., 2004) [1].

In agreement with the finding of Angel *et al*. (2004) [1] our study also revealed that an optimum time period of incubation and a suitable substrate plays a major role in the production of OTA. Thus, the substrate de-oiled sunflower cake can be used as a better medium than cereals (polished rice, maize and pearl millet) to support OTA production. As mycotoxin production is known to occur under favourable and stress conditions, (Magan *et al*., 2002) [19], the specific reasons which directed towards the OTA production in this study can be speculated. Eventhough many fungal species have simple nutrient utilization (Kokkonen *et al*., 2005) [14], the better yield obtained in this study may be due to the presence of balanced amino acid composition (Morrison *et al*., 1953) with all the essential amino acids distributed in it naturally required for the growth. Also the present findings of the study demonstrate the distinctive effect of the substrate on the secondary metabolism of the fungi examined. The toxin production might have enhanced due to the increased primary metabolism during the incubation period. The secondary metabolism might have occurred later (Kokkonen *et al*., 2005) [14]. Hence, to understand better and carry out research related to OTA production and for the characterization of OTA producing *A. ochraceus* isolates, de-oiled sunflower cake can be an apt and better choice of substrate for the production of toxins in laboratory.

Fig 1: Screening the substrates for the production of OTA

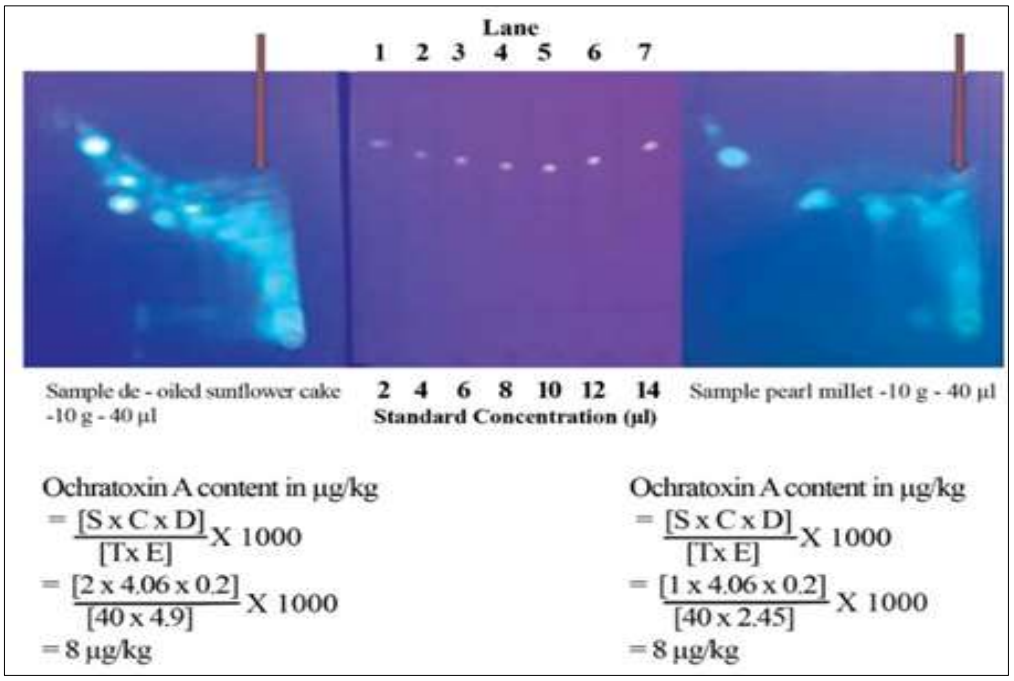

Fig 2: Production of OTA in de-oiled sunflower cake and pearl millet on 7 days of incubation

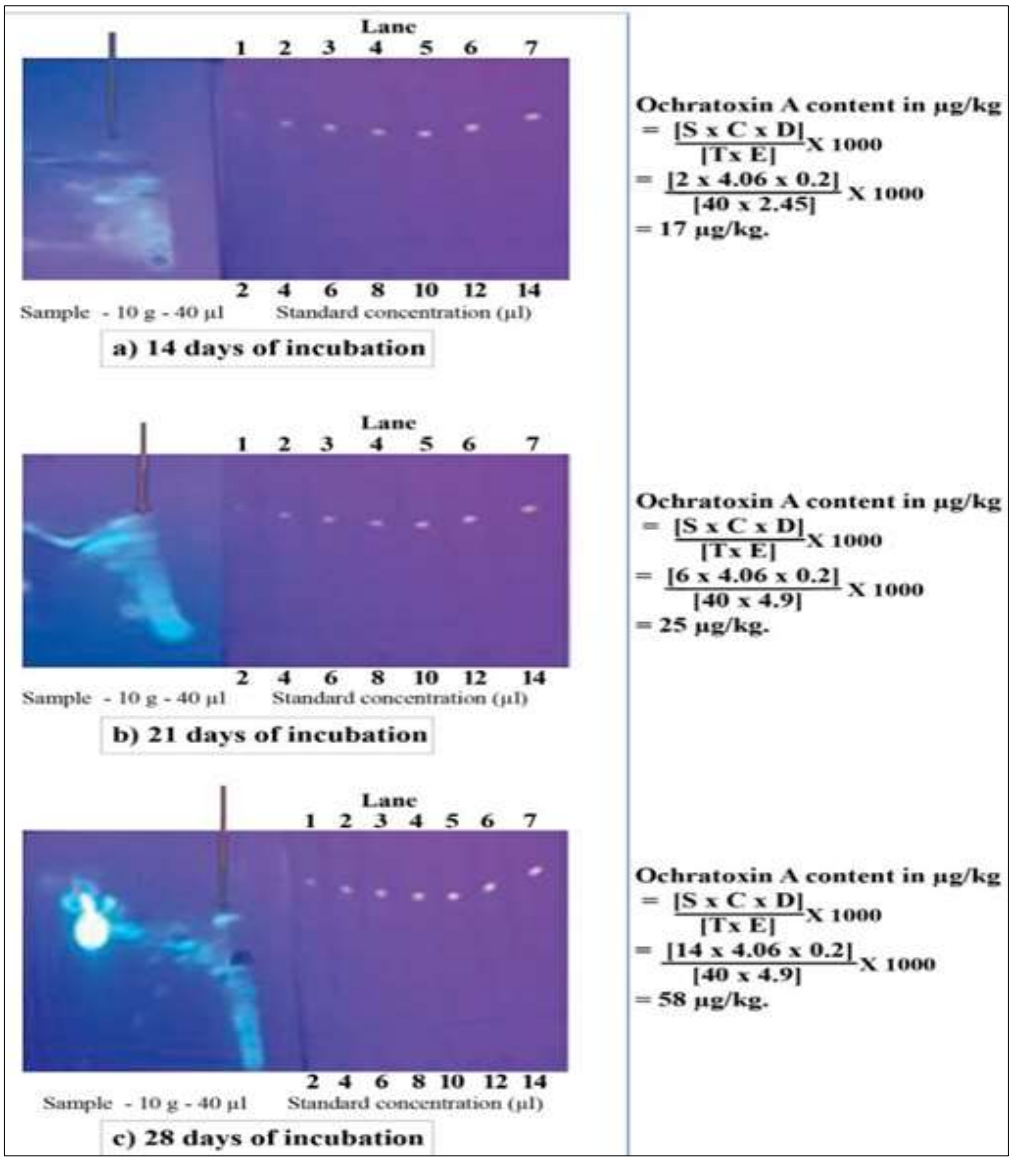

Fig 3: Estimation of OTA at different times of incubation in de-oiled sunflower cake

4. Conclusion

It was concluded that de-oiled sunflower cake was the best suitable substrate for OTA production among the cereals and other cakes experimented. The level of toxin detected on 28 days of incubation was higher than short duration attempted (7 days) suggesting the time (duration) needed to produce substantial quantity of OTA under laboratory conditions was found to be much longer than a week. However, the study to enhance the growth of *Aspergillus ochraceus* and OTA production in de-oiled sunflower cake has to be further strengthened to find out whether further high levels of OTA could be harvested by manipulation of temperature, humidity, duration and even substrate quantity. Nevertheless, the present finding that de-oiled sunflower cake was a better substrate for OTA production by *Aspergillus ochraceus* has given a hope for the enhanced production for experimental purpose. It also becomes very clear that a substrate enhancing the growth under experimental conditions are more vulnerable for natural contamination in the field. Hence, the risk factor associated with the usage of the de-oiled sunflower cake in the animal feed need to be taken into account as the level of the toxin is closely approaching the permissible level as recommended by European Commission (ECR2006/575/EC of 0.1 mg/kg) which may increase if the incubation is prolonged and this may happen in the field so rapidly. So, it is highly essential to sensitize the feed manufacturers accordingly while formulating feeds so as to enable them to adopt suitable control measures to avoid health risks for livestock and poultry and for conducting toxicological studies in poultry. Moreover, the toxin production is a strain specific characteristic as far as *A. ochraceus* is concerned which can be ascertained by enhancing the growth of the fungal culture in the substrate.

5. Acknowledgment

The authors pay utmost gratefulness to TANUVAS for the financial support rendered for the successful completion of the research work. The authors are also highly thankful to the Dean of our college for the constant encouragement and support. The authors express their heartfelt sincere thanks to the staff of Animal Feed Analytical and Quality Assurance Laboratory for their valuable contribution and cooperation during the entire course of the study.

6. Conflict of Interest

The authors express no conflict of interest.

7. Funding

There is no external funding for the study.

8. Data availability

All the relevant data are included for publication

9. Authors contribution

Conceptualization – Sakthi Priya Muthusamy and Jagadeeswaran Appusamy
Investigations – Sakthi Priya Muthusamy, Jagadeeswaran Appusamy and Natarajan Amirthalingam
Methodology - Sakthi Priya Muthusamy, Jagadeeswaran Appusamy and Natarajan Amirthalingam
Writing, Review and editing - Sakthi Priya Muthusamy, Jagadeeswaran Appusamy and Natarajan Amirthalingam
All authors have read and agreed to the published version of the manuscript.

10. Reference

1. Ángel M, Gabriela G, Joan MS, Rufino M, Misericordia J. Bee Pollen, a Substrate that Stimulates OTA production by *Aspergillus ochraceus* Wilh. System. Appl. Microbiol. 2004;27:261-267.
2. AOAC. Official methods of analysis. 6th ed. Association of Official Analytical Chemists, Washington, DC; c1995.
3. Begun F, Samajpati N. Mycotoxin production on rice, pulses and oilseeds. Natur wissens chaften. 2000;87:257-277.
4. Betina V. Bioactive molecules. Mycotoxins, Chemical, Biological and Environmental aspects. Elsevier Science, Amsterdam; c1989, 9.
5. Bhat R, Rai RV, Karim AA. Mycotoxins in food and feed: present status and future concerns. Comp. Re. Food. Sci. Food. Sa. 2010;9:1-175.
6. Ciegler A. Bioproduction of OTA and penicillic acid by members of the *Aspergillus ochraceus* group. Can. J Microbiol. 1972;18:631-636.
7. Coulombe RA, Coulombe Jr. Biological action of mycotoxins. Journal of Dairy Science. 1993;76:880-891.
8. Donmez- Altunta H, Hamurcu Z, Imamoglu N, Liman BC. Effects of ochratoxin A on micronucleus frequency in human lymphocytes. Nahrung/Food. 2003;47:33-35.
9. Filtenborg O, Frisvad JC, Samson RA. Specific association of fungi to foods and influence of physical environmental factors. In: Samson, R.A., Hoekstra, E.S., Frisvad, J.C., Filtenborg, O. (Eds). Introduction to food and airborne fungi, 6th ed. Central bureauvoor Schimmel cultures. Ultrecht; c2000. p. 306-320.
10. Filtenborg O, Frisvad JC, Thrane U. Moulds in food spoilage. Int. J Food. Mib. 1996;33:85-102.
11. Hendry KM, Cole EC. A review of mycotoxins in indoor air. J Toxicol Environ Health. 1993;38:183-198.
12. Hesseltine CW, Vanergraft EE, Fennell DI, Smith ML, Shotwell OL. *Aspergilli* as ochratoxin producers. Mycologia. 1972;64:539-550.
13. Jand SK, Kaur P, Sharma NS. Mycoses and mycotoxicosis in poultry: A review. Indian J Anim. Sci. 2005;75(4):465-476.
14. Kokkonen M, Jestoi M, Rizzo A. The effect of substrate on mycotoxin roduction of selected enicillium strains. Int. J Food. Mib. 2005;99:207-214.
15. Lacey J. Natural occurrence of mycotoxins in growing and conserved forage crops. In: Smith, J.E., Henderson, R.E., (eds) Mycotoxins and animal foods. CRC Press, Boca Raton; c1991. p. 363-397.
16. Lai M, Semeniuk G, Hesseltine CW. Conditions for production of OTA by *Aspergillus* species in synthetic medium. Appl. Microbiol. 1970;19:542-544.
17. Leszkowicz AP, Manderville RA. Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. Mol Nutr Food Res. 2007;51(1):61-99.
18. Lindblad M, Johnsson P, Johnsson N, Lindqvist R, Olsen M. Predicting non-compliant levels of OTA in cereal grain from *Penicillium verrucosum* counts. J Appl Microbiol. 2004;97:609-616.
19. Magan N, Hope R, Colleate A, Baxter ES. Relationship between growth and mycotoxin production by *Fusarium* species, biocides and environment. E. J Plant. Pathol. 2002;108:685-690.

20. Morrison AB, Clandinin DR, Robblee AR. The effects of processing variables on the nutritive value of sunflower seed oil meal. Poultry Science. 1953;32(3):492-496.
21. Rao CY. Toxigenic fungi in the indoor environment. In: Spengler JD, Samset JM, McCarthy JS (eds). Indoor air quality handbook. McGraw-Hill, Washington, DC; c2001. p. 1-17.
22. Shotwell OL, Hesseltine CW, Stabblefield RD, Sorenson GW. Production of aflatoxin on rice. Appl. Microbiol. 1966;14:425-428.
23. Trenk HL, Butz ME, Chu FS. Production of Ochratoxins in different cereal products by *Aspergillus ochraceus.* Applied Microbiology; c1971. p. 1032-1035.
24. Trung TS, Bailly JD, Querin A, Les Bars P, Guerre P. Fungal contamination of rice from South Vietnam, mycotoxigenesis of selected strains and residues in rice. Rev. Med. Vet. 2001;152:555-560.
25. Tsubouchi H, Terada H, Yamamoto K, Hisada K, Sakabe Y. Caffeine degradation and increased ochratoxin production by toxigenic strains of *Aspergillus ochraceus* isolated from green coffee beans. Mycopathologia. 1995;90:181-186.
26. Van der Merwe KJ, Steyn PS, Fourie L. Mycotoxins. Part II. The constitution of ochratoxin A, B and C metabolites of *Aspergillus ochraceus* Wilh. J Chem. Soc; c1965a. p. 7083-7088.
27. Van der Merwe KJ, Steyn PS, Fourie L. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus. Wilh. Nature* (London). 1965b;205:1112-1113.
28. Varga J, Kevei É, Tóth B, Kozakiewicz Z, Hoekstra RF. Molecular analysis of variability within the toxigenic *Aspergillus ochraceus* species. Can. J Microbiol. 2000;46:593-599.
29. Varga J, Kevei E, Rinyu E, Téren J, Kozakiewicz Z. Ochratoxin production by *Aspergillus* species. Appl. Environ. Microbiol. 1996;62:4461-4464.
30. Varga J, Rigó K, Lamper C, Téren J, Szabó G. Kinetics of OTA production in different *Aspergillus* species. Acta Biologica Hungarica. 2002;53:381-388.
31. Viquez OM, Castell Perez ME, Shelby RA, Brown G. Aflatoxin contamination in corn samples due to environmental conditions, aflatoxin producing strains, and nutrients in grain grown in Costa Rica. J Agric. Food. Chem. 1994;42:2551-2555.